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Prospecting the rumen protozoa for novel enzymes using metagenomic techniques

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Context

Protozoa, along with fungi, represent the rumen eukaryotome and account for up to 50% of microbial biomass[1]. Nonetheless, they are often overlooked. It is inferred that rumen protozoa play a role in lipid, protein and fibre metabolism, however, few of these enzymes have been identified and characterised[2]. Enzymes previously isolated from the rumen microbiome have shown great potential for application in industry (biodiesel manufacture, the food industry, washing detergents etc.) suggesting a wealth of untapped, novel activity in the rumen eukaryotes.

Study Aim

To explore the rumen protozoa and their enzymes using metagenomic and metatranscriptomic techniques.

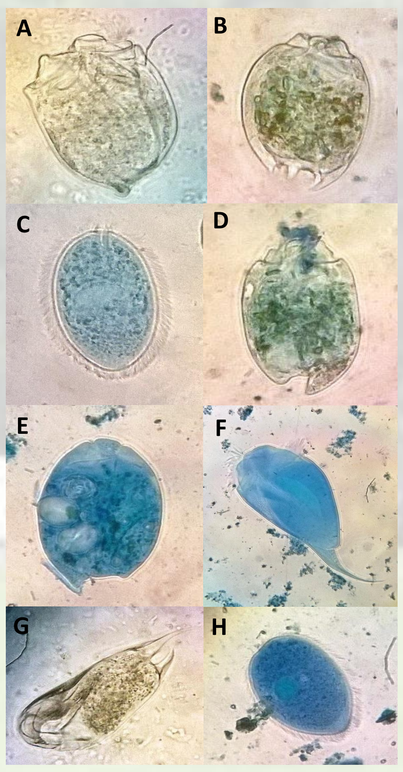

Fig.1: A: Entodinium; B: Diplodinium; C: Isotrichia; D: Euplodinium; E: Entodinium; F: Epidinium; G: Epidinium; H: Dasytrichia.

Fig.2: Separation of the protozoa from rumen fluid in pear-shaped, 1L burette and 0.5g glucose.

Materials and Methods

Rumen samples were taken from three fistulated, non-lactating Friesian-Holstein cows and pooled. Protozoa were separated by addition of glucose (Fig 2) and subjected to several washes using Coleman's buffer and centrifugation. RNA was extracted (FastRNA Pro Soil-Direct kit), PolyA purified (Poly(A)Purist MAG kit), DNase treated (TURBO DNase) and reverse transcribed using LDPCR (SMART cDNA Library kit). cDNA was digested using SfiI (A & B) enzymes, size fractionated, ligated into the λTriplEx2 vector and packaged into λ phage (MaxPlax Lambda extract). The primary library was titred and amplified, then screened under X-Gal/IPTG to determine recombinant: non-recombinant ratio (Fig 3). Library inserts were characterised using vector encoded primers. The library was screened for cellulase activity using carboxymethyl cellulose (CMC) agar with post staining using Congo Red. The library was also screened using Spirit Blue and Egg yolk agar. Sequencing of the library as a whole is being conducted using the HiSeq 2500 system.

Acknowledgements

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MICROBIOLOGY SOCIETY

References:
[1] Belanche. A, Abecia. A, Holtrop. G (2011), *J. Anim. Sci.* **89**: 4163-4174
[2] Qi. M, O'Toole. P, Barboza. P. S (2011), *PLOS One*, **6**(5)

Results

- The primary library showed recombinant (white): non-recombinant (blue) plaque ratio of 100:1 and titre= 6.5 x 10^6 pfu/mL (fig 3).
- The amplified library showed a ratio of 500:1 recombinants: non-recombinants and titre = 2.53 x 10^8 pfu/mL (fig 3)
- Inserts varied in size and ~50% showed homology with known protozoal genes in (fig 5)
- Six plaques showed CMCase activity (fig 6).
- Screening with Egg yolk and Spirit blue agar was unsuccessful due to the sensitive nature of phage-based libraries.

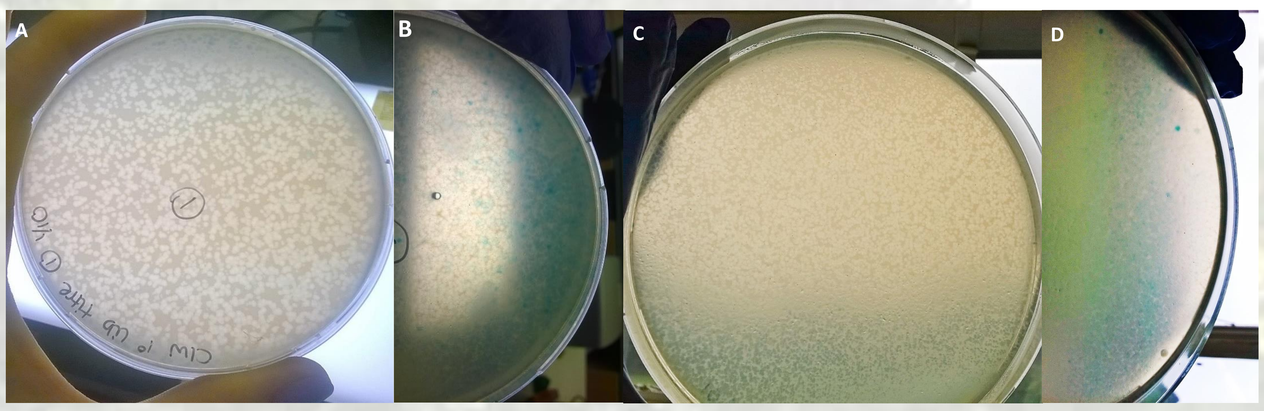

Fig 3: A-B: Primary library plated on *E.coli* lawn with X-gal and IPTG showing blue and white plaques (ratio approx. 1:100). **C-D:** Amplified library plated in the same way showing blue and white plaques (ratio approx. 1:500).

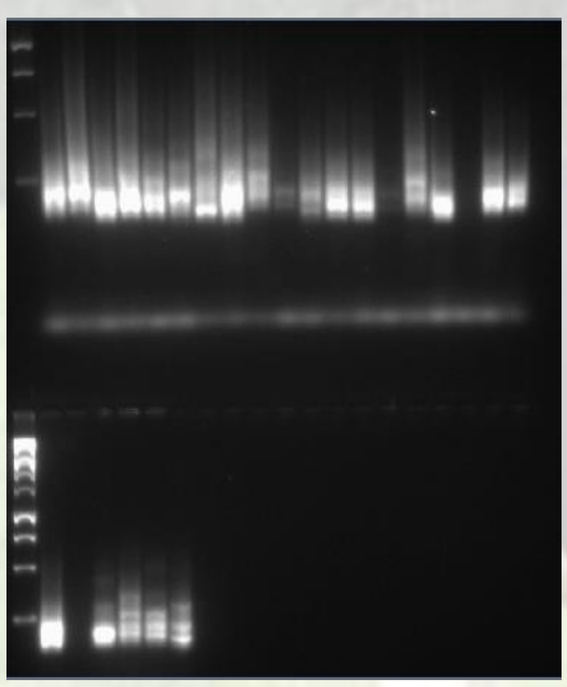

Fig 5: Insert characterisation using PCR with vector encoded primers and 2% gel electrophoresis. 1Kb size standard in first lanes.

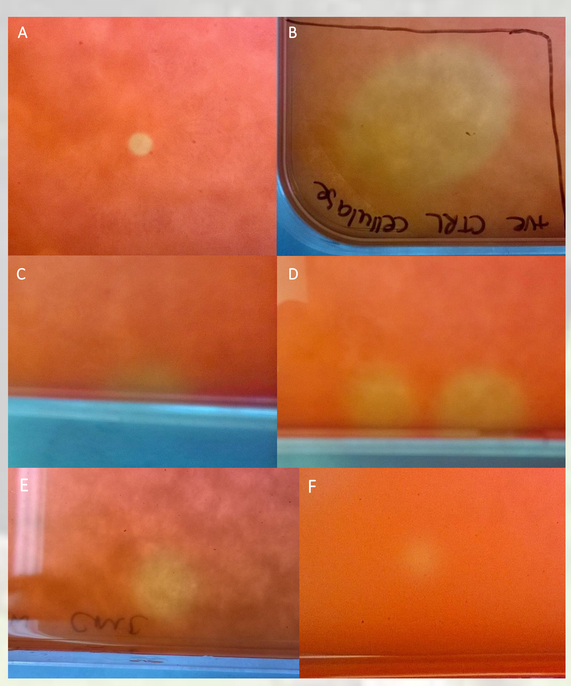

Fig 6: Amplified library plated on *E.coli* lawn with CMC overlay and post-staining with Congo red.

Discussion

The SMART cDNA library construction kit worked well using low amounts of starting RNA, it provided adequately sized inserts and good recombinant: non-recombinant ratios. The main drawback of the kit being that *in vivo* excision is not possible, which would have allowed accurate sequencing of inserts. Insert sequencing showed matches to partial sequences from *Epidinium* sp, *Epispathidium* sp, *Entodinium* sp, *Spathidium* sp, *Dileptus* sp and several uncultured rumen protozoa.

When screened for CMCase activity, six positives were identified. These plaques were gored and applied to secondary screening as well as Sanger sequenced – however, this was not successful. Possibly due to the sensitive nature of phage screening using Spirit blue and Egg yolk agar was also unsuccessful as indicated by the lack of plaque formation.

The majority of sequencing runs gave mixed, overlapping results due to the sheer number of phage in each plaque. Positive areas on the CMC agar covered approx. 10 plaques each, in turn each plaque can contain over 10,000 individual phage.

Sequencing of the cDNA used in the library is underway using the HiSeq 2500 platform. The HiSeq system will allow deep sequencing to identify both functionality and taxonomy. This sequence-based approach will allow identification of novel genes and subsequent cloning and characterisation.